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Phenotypic plasticity of floral volatiles in response to increasing drought stress

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- **Background and Aims** Flowers emit a wide range of volatile compounds which can be critically important to interactions with pollinators or herbivores. Yet most studies of how the environment influences plant volatiles focus on leaf emissions, with little known about abiotic sources of variation in floral volatiles. Understanding phenotypic plasticity in floral volatile emissions has become increasingly important with globally increasing temperatures and changes in drought frequency and severity. Here quantitative relationships of floral volatile emissions to soil water content were analysed.
- **Methods** Plants of the sub-alpine herb *Ipomopsis aggregata* and hybrids with its closest congener were subjected to a progressive dry down, mimicking the range of soil moistures experienced in the field. Floral volatiles and leaf gas exchange were measured at four time points during the drought.
- **Key Results** As the soil dried, floral volatile emissions increased overall and changed in composition, from more 1,3-octadiene and benzyl alcohol to higher representation of some terpenes. Emissions of individual compounds were not linearly related to volumetric water content in the soil. The dominant compound, the monoterpene α-pinene, made up the highest percentage of the scent mixture when soil moisture was intermediate. In contrast, emission of the sesquiterpene (*E,E*)-α-farnesene accelerated as the drought became more intense. Changes in floral volatiles did not track the time course of changes in photosynthetic rate or stomatal conductance.
- **Conclusions** This study shows responses of specific floral volatile organic compounds to soil moisture. The non-linear responses furthermore suggest that extreme droughts may have impacts that are not predictable from milder droughts. Floral volatiles are likely to change seasonally with early summer droughts in the Rocky Mountains, as well as over years as snowmelt becomes progressively earlier. Changes in water availability may have impacts on plant–animal interactions that are mediated through non-linear changes in floral volatiles.

Key words: Drought, floral scent, *Ipomopsis aggregata*, *Ipomopsis tenuituba*, phenotypic plasticity, VOC.

INTRODUCTION

Angiosperms show remarkable diversity not only in size, shape and colour of flowers, but also in scent, with >1700 volatile organic compounds (VOCs) emitted by flowers (Knudsen *et al.*, 2006). These compounds can influence the attraction of animal pollinators or herbivores (Raguso, 2008; Kessler *et al.*, 2013). Like other aspects of the floral phenotype, scent experiences natural selection mediated by pollinators (Schiestl *et al.*, 2011; Parachnowitsch *et al.*, 2012), and differences in scent between closely related plant species can contribute to reproductive isolation and speciation (Bischoff *et al.*, 2015; Gervasi and Schiestl, 2016). Variation in floral scent composition is commonly observed not only between species but also within a plant species (Delle-Vedove *et al.*, 2017). With the exception of comparisons across times of day (Dötterl *et al.*, 2005; Jürgens *et al.*, 2014; Campbell *et al.*, 2016; Chapurlat *et al.*, 2018) however, remarkably little is known about sources of intraspecific variation in floral VOCs, including the roles of specific environmental conditions (Raguso *et al.*, 2015). Understanding the potential for such phenotypic plasticity in response to the environment, and whether the plasticity is adaptive (Anderson *et al.*, 2012), has become increasingly important with recent climate change (IPCC, 2014).

Most research on environmental sensitivity of VOC emissions has focused not on flowers but on leaf emissions by trees, and often in the context of atmospheric processes (e.g. Niinemets, 2010; Trowbridge *et al.*, 2014; Seco *et al.*, 2015; Eller *et al.*, 2016). Floral VOCs could also potentially respond to abiotic conditions such as temperature, soil nutrients or soil water availability (Majetic *et al.*, 2009; Yuan *et al.*, 2009; Burkle and Runyon, 2016). Even quantitative changes in these emissions could influence plant–animal interactions. Some insects respond not only to the presence of particular volatile compounds but also to the dosage of the volatile (Galen *et al.*, 2011), which can even shift from an attractant to a repellant (Terry *et al.*, 2007). Phenotypic plasticity in floral volatiles thereby has high potential to influence ecological processes, including species interactions such as pollination and floral herbivory. Yet studies of phenotypic plasticity in floral volatiles remain rare (Farré-Armengol *et al.*, 2014; Burkle and Runyon, 2016; Friberg *et al.*, 2017).

In this study, we focus on phenotypic plasticity in response to water availability. Soil water is likely to change with future climate change, even in the absence of changes in precipitation, as warmer temperatures increase levels of evapotranspiration and can accelerate snowmelt (IPCC, 2014). Simulations often project declines in surface water availability even in regions, such as the south-western USA, where projections for precipitation itself are uncertain (Seager *et al.*, 2012). How soil moisture influences floral volatiles is poorly understood, with just one set of studies comparing floral emissions under dry vs. wet conditions in herbaceous plants in the northern Rocky Mountains of North America (Burkle and Runyon, 2016; Glenny *et al.*, 2018). In that case, responses of floral VOCs varied across species from diverse angiosperm families; total volatiles increased with drought in *Campanula rotundifolia* (Campanulaceae), *Phacelia hastata* (Hydrophyllaceae) and *Potentilla recta* (Rosaceae), but not in *Heterotheca villosa* (Asteraceae). Such species to species variation might be explained by the severity of a drought and how a given plant species responds to a particular level of water availability. For leaves of some woody plants, mild drought stress can reduce stomatal conductance and increase isoprene emission, whereas prolonged severe drought stress often causes a reduction in emission, in part due to reduction of enzyme activity needed for biosynthesis (reviewed in Copolovici and Niinements, 2016). Similar increases followed by decreases with further drought have been reported for monoterpene emissions from leaves (Wu *et al.*, 2015). For floral volatiles, we are unaware of any studies that have examined quantitative responses to drought beyond comparing snapshot responses to the two categories of dry and wet. Leaf studies will not necessarily translate to floral volatiles, as leaf emissions may be more closely coupled to photosynthetic processes and availability of intermediates for biosynthesis in the leaves (Niinemets *et al.*, 2004), and different plant organs are involved in emissions. Floral emissions can come from a diverse set of floral parts (Knauer and Schiestl, 2015) or even rewards such as pollen or nectar (Raguso, 2004).

We subjected plants of the herb *Ipomopsis aggregata* (Polemoniaceae), from sites in Colorado, and hybrids of that species with its close congener *I. tenuituba* (Porter *et al.*, 2010) to progressively increasing drought stress and examined the impacts on emission of floral volatiles and leaf-level photosynthetic gas exchange. By using a larger sample size of individual plants than in the one set of previous studies and examining responses over the course of a progressive dry down, we were able to characterize, for the first time, quantitative responses of specific floral volatiles to soil moisture. We also measured concurrent responses of leaf gas exchange to drought, even though such responses have been studied before in *Ipomopsis* (Campbell *et al.*, 2010; Campbell and Wendlandt, 2013), for two reasons. First, they allow a direct comparison with the time course of volatile emissions to test for associations between gas exchange and emission, as seen under some conditions for monoterpenes in leaves (Nogues *et al.*, 2015; Eller *et al.*, 2016). Secondly, the photosynthetic rate and stomatal conductance provide an index of plant stress at the time of volatile measurements. Floral volatiles in *I. aggregata*, *I. tenuituba* and natural hybrids have been shown to influence attraction of hawkmoth pollinators and the potential for reproductive isolation (Bischoff *et al.*, 2015), and probably also influence the oviposition rate by a pre-dispersal fly seed predator (Brody, 1992; Bischoff *et al.*, 2014). We asked the following questions. (1) How do the total emission and composition of floral volatiles change with increasing drought stress? Emissions could be highly plastic, or alternatively might be regulated to maintain more consistent levels. (2) For floral volatiles that respond to drought stress, what is the form of the relationship to soil moisture? (3) Do changes in floral volatiles track the time course of changes in leaf photosynthetic rate or stomatal conductance?

MATERIALS AND METHODS

Study system

Our experiments used potted plants of *Ipomopsis aggregata* subsp. *aggregata* (eight plants in 2015 and ten plants in 2017) and F_2 hybrid plants originating from a cross between *I. aggregata* subsp. *aggregata* and *I. tenuituba* subsp. *tenuituba* (six plants in 2017). We included hybrids for two reasons. First, their inclusion enlarged the range of plant volatile responses studied, as scent blends emitted by natural hybrids and *I. aggregata* differ (Bischoff *et al.*, 2014). Secondly, advanced generation hybrids between these species, including F_2, occur commonly in natural hybrid zones in the south-western USA (Wu and Campbell, 2005; Aldridge and Campbell, 2009). An individual plant of these taxa typically produces a single flowering stalk with multiple flowers (average near 80 for *I. aggregata*) over the course of a 4–5 week blooming period (Campbell, 1989).

Flowering individuals of *I. aggregata* were obtained from sub-alpine meadows approx. 1 km south of the Rocky Mountain Biological Laboratory, Gunnison County, CO, USA. Following transplantation into 6 inch plastic pots with native soil from the site, they were moved into an outdoor WeatherPort screenhouse (Weatherport, Delta, CO, USA) and watered ad lib for 2 weeks prior to use in an experiment. Previous work has demonstrated recovery from transplant shock over that time interval (Campbell *et al.*, 2010). The grandparents of the F_2 hybrids were obtained from Grizzly Ridge, Montrose County, CO, USA (see site description in Aldridge and Campbell, 2009). They were crossed in 2012 to produce F_1 hybrid seeds, and the F_1 were crossed in 2014 to produce F_2 seeds. Plants of these species of *Ipomopsis* are monocarpic, and the F_2 individuals we used flowered in 2017. Crosses were done in both directions, and of the six F_2 that we used, half (three) had maternally inherited cytoplasmic genes from *I. aggregata.* The hybrid plants had been maintained in 6 inch plastic pots for their entire life time, inside a Weatherport during summers and sunk into the ground to overwinter, as in previous studies (Campbell and Waser, 2001). Complete NPK fertilizer was applied to all plants once per summer.

Dry-down experiments

To examine effects of drought on floral volatiles, in both 2015 and 2017 we conducted dry-down experiments in which the potted plants were allowed to dry out progressively after withholding irrigation. Our experiment generated a range of soil moistures (see the Results) typical of those experienced by local natural populations of *Ipomopsis* [e.g. range of 2.7–26 % volumetric water content (vwc); Campbell and Wu, 2013; Waser and Price, 2016]. On day zero of the experiment, plants were watered to saturate the soil, and then the plants were allowed to dry progressively. Both floral volatiles and leaf-level gas exchange were measured at four time points on each plant (start, early, mid and late drought). These four time points corresponded to day 1, 5, 10 and 12 in 2015 and to day 1, 5, 8 or 10, and 13 in 2017. To check for the ability of plants to recover from the drought stress and establish that changes in volatiles were not a consequence of irreversible plant decline, in 2015 we also measured leaf-level gas exchange on day 17 after re-watering the plants on day 13. Plants were measured in a randomized order, with the same order used for each date. Temperature did not change in any systematic way over the course of these experiments. Linear regressions of maximum daily air temperature on a particular date were not significant for 3 August to 19 August in 2015 ($P = 0.34$) or for 19 July to 31 July in 2017 when they ranged only from 22 °C on day 8 to 24 °C on day 1 ($P = 0.45$).

Volatile measurements

On each day, we collected a scent sample from a single flower on each plant using dynamic headspace methods. Sampling from a single flower ensured that volatiles are emitted from a flower rather than structural portions of the inflorescences. We followed the general methods in Bischoff *et al.* (2014), with the exception that the scent trap (consisting of a microvial made from a capillary tube with internal diameter of 1 mm and sealed with silanized quartz wool) was filled with 5 mg of Tenax TA® as the trapping agent. Briefly, a single flower was enclosed in a Reynold's oven bag heat sealed to dimensions of 7 × 10 cm. Headspace volatiles were allowed to equilibrate for 30 min. The scent trap was then inserted into the bag, with the other end connected with tubing to a micro air sampler (Supelco PAS-500) at a flow rate of 100 mL min^{-1} for 15 min. We also took two kinds of ambient controls, an air sample and a leaf sample that were collected for the same amount of time, each day that volatiles were sampled. Air controls used empty Reynold's oven bags with the same dimension as used for the flower samples. Leaf controls were taken by enclosing a single leaf in the oven bag. Each plant was moved to an outdoor bench immediately prior to sampling. All sampling was done between 09.00 and 12.00 h, as for daytime samples in previous studies of these species (Bischoff *et al.*, 2014). Some volatile samples from 2015 could not be analysed because of loss during attempts to use an older gas chromatography–mass spectrometry (GC-MS) instrument prior to obtaining new equipment in 2017. In total, we analysed 97 floral samples and 15 ambient controls, including both air and leaf samples.

The scent traps were analysed using thermal desorption GC-MS. We used a Markes Ultra autoloading system connected to a Markes Unity-xr to introduce the volatiles to a Shimadzu GC-MS QP2020 at the Rocky Mountain Biological Laboratory. Each capillary tube scent trap was put inside a stainless steel Markes sampling tube and loaded into the autosampler to undergo a two stage desorption. The primary desorption was for 5 min at 250 °C. The sample was then trapped at a low temperature of 25 °C on a Tenax-filled cold trap, followed by heating rapidly to 200 °C for 3 min. The GC oven cycle was held for 2 min at 40 °C to retrap volatiles, then ramped at 10 °C min^{-1} to 250 °C and at 30 °C min^{-1} to 275 °C, with a final hold for 3 min. We used ultra-high purity (99.999 %) helium as the carrier gas and an Rtx-5MS column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness).

Volatiles were tentatively identified using Shimadzu GCMS Postrun software to find the top match in the NIST14 library (National Institute of Standards and Technology), with a minimum similarity of 70 %, and confirmed by comparing retention times and mass spectra with authentic standards, when available. Peak areas were exported to Excel. We wrote a program in R 3.4.1 to compare peak areas in floral samples with those in ambient air controls to eliminate contaminants. In the R program, we first found compounds for which the mean peak area in the floral samples exceeded three times the mean in ambient controls (which was often zero). For those compounds, we then performed *t*-tests with a false discovery rate of 5 % to control for multiple comparisons. A compound was retained in the final data set if it passed that stringent statistical criterion and had been reported as a known floral volatile in www.pherobase.com or in a comprehensive review (Knudsen *et al.*, 2006). Eleven compounds met those requirements, of which eight had previously been reported as floral volatiles specifically in *I. aggregata* or *I. tenuituba* from similar sites (Bischoff *et al.*, 2014). We added 17 other compounds that did not meet the strict statistical criterion but had higher averages for floral samples than ambient controls (15 of those 17 were never seen in air controls; Supplementary Data Table S1) and had been previously reported as *I. aggregata* or *I. tenuituba* floral volatiles (Bischoff *et al.*, 2014). All compounds were found in multiple floral samples (mean = 28 floral samples, range = 2–96 floral samples). Volatiles were quantified by running authentic standards in several amounts (0.3, 3, 30 and 300 ng), with 2–4 replicates per dosage, to find a regression relationship between known amount and peak area. We ran eight standards and used either the same compound or a standard from the same chemical class (aliphatics, benzenoids, monoterpenes, sesquiterpenes or nitrogen compounds) to quantify emissions for a particular compound. Amounts were converted to ng h^{-1} by multiplying the amount emittted during the 45 min sampling period by 4/3, as the total sampling period, including bagging and pumping, was 45 min (Bischoff *et al.*, 2014). Our method of sampling volatiles onto scent-absorbing polymers that are thermally desorbed can be considered quantitative. It gave highly repeatable results for known standards, with R^2 for the relationship between amount and peak area = 0.92–0.97.

Leaf-level gas exchange

On each morning that volatiles were measured, we also measured leaf-level gas exchange. Photosynthetic rate (assimilation A) and stomatal conductance (g_s) were measured for each plant just prior to measurement of its floral volatiles. We used a Li-Cor 6400 photosynthesis system (Li-Cor, Lincoln, NE, USA). Leaf chamber conditions were standardized with a temperature of 25 °C, photosynthetically active radiation (PAR) at

1800 µmol m^{-2} s^{-1} to give light-saturated photosynthesis (Wu and Campbell, 2006; Campbell *et al.*, 2010), and CO_2 concentration of 400 ppm to reflect recent atmospheric conditions. Two measurements were recorded on a single leaf, and the two measurements were averaged prior to analysis. Following gas exchange measurements, the measured leaves were scanned to obtain leaf area using ImageJ software (National Institutes of Health freeware). All values are reported on a per leaf area basis. We were not able to measure gas exchange on day 13 in 2017. The vwc of the soil was measured in each pot immediately after each measurement. In 2017, we used a Hydrosense II soil moisture sensor (Campbell Scientific, Edmonton, Canada). Data from 2015 were taken with an older model Hydrosense soil moisture sensor, and we calibrated the two instruments against each other, converting the 2015 measurements based on regression ($R^2 = 0.96$, $n = 35$ measurements with both instruments).

Statistical analyses

To determine how the total emission and composition of floral volatiles changed with drought stress (question 1), we performed multivariate analysis of variance (MANOVA), using both standard parametric methods and permutational analysis of variance (PERMANOVA; Anderson, 2001), and a canonical discriminant analysis. Prior to analysis, peak areas were log transformed (after adding 1) to reduce the tendency for variance to increase with the mean and the skew due to zero-inflated data. The MANOVA specified the individual plant as a block along with the fixed factor of time point (start, early, mid or late drought). Use of MANOVA accounted for potential correlations among responses of different volatiles. Use of plant as a blocking factor allowed for correlations in responses of a given volatile across date, but assumed they were equally correlated regardless of the time interval. A doubly multivariate repeated analysis allowing for other correlation structures was not used because of limited and unequal sample sizes. For the standard MANOVA, Type III SS in SAS v9.3 was employed because of unequal sample sizes. Because residuals were not normally distributed, we checked significance levels with a PERMANOVA, also with factors of plant and time point, using the adonis function in the Vegan package of R (ver. 3.4.1). The canonical discriminant analysis (Proc Candisc in SAS v9.3) found the linear combinations of scent variables that explained the greatest variance among means for the four time points (start, early, mid or late drought). Although the two types of plants (*I. aggregata* and F_2 hybrid) differed in composition of floral volatiles, those emissions responded similarly to drought, as evidenced by lack of detectable plant type × time interactions (analysis is shown in Supplementary Data Table S2).

We analysed the quantitative form of the relationship between volatile emissions and drought (question 2) in two ways. First, we used Tukey post-hoc comparisons to identify the time points in the drought that differed in the randomized block ANOVAs on log-transformed values, with individual plant as a blocking factor. Separate ANOVAs were performed for emission of each individual volatile, total VOC emission and the untransformed proportion of the mixture made up by the largest constituent, α-pinene. Secondly, for each of the four compounds with emissions that differed significantly across days and that correlated significantly with vwc in the soil (Pearson's correlation, $P < 0.05$), we examined the form of the relationship of emission rate to vwc with non-parametric regression methods. For many of the compounds, variance in emissions changed greatly with vwc, violating an assumption of traditional regression analysis. For that reason and because it was unclear what shape relationship to expect, we employed local polynomic regression (LOESS), choosing the smoothed curve that minimized AICC, a variant of Akaike's information criterion (Hurvich *et al.*, 1998). This analysis was implemented in Proc LOESS of SAS v.9.3.

To characterize the change in photosynthetic rate (assimilation rate = A) and stomatal conductance (g_s) with soil moisture (question 3), we employed analyses of covariance (ANCOVAs). The factor was type of plant (*I. aggregata* or hybrid), and continuous variables were vwc and the squared difference of vwc from its mean, to allow for a quadratic relationship (as seen in Campbell *et al.*, 2010). Since relationships of volatiles to photosynthetic parameters can change depending upon the overall degree of drought stress (Eller *et al.*, 2016), we tested for their relationships to A or g_s using ANCOVA, with the factor of day, A (or g_s), and the interaction of day with A (or g_s). We ran these tests for log-transformed total volatiles and also for all four of the volatiles whose emissions correlated with soil moisture, correcting the P-values using a false discovery rate for four tests. None of the interactions was statistically significant, so we followed up with standard ANCOVAs that did not include the interaction term. ANCOVAs were run with Proc GLM in SAS v9.3, using type III SS.

RESULTS

Over the course of the dry-down experiment, mean vwc in the soil changed from 16.6 % (s.e. = 1.3 %) on day 1, to 7.4 % (s.e = 0.7 %) on day 5, to 3.7 % (s.e. = 0.4%) on days 8–10 and to 2.5 % (s.e. = 0.2 %) on days 12–13. The most abundant floral volatile was α-pinene, with ten compounds each making up at least 1 % of the scent mixture in either *I. aggregata* or the hybrids (Supplementary Data Table S2). Because rare compounds can influence the behaviour of pollinators and herbivores (Bischoff *et al.*, 2015), we included all floral compounds regardless of percentage in our statistical analyses. The MANOVA on the scent compounds revealed significant changes across the time points in the drought (Wilks' lambda = 0.112 and $P = 0.0044$, PERMANOVA $P = 0.011$) as well as a main effect of individual plant ($P < 0.0001$, PERMANOVA $P < 0.001$). In a canonical discriminant analysis separating the four time points, the first two canonical functions explained 85 % of the variance. The first canonical discriminant function (CDF1) was highly correlated with group membership (canonical correlation = 0.70, $P = 0.0037$) and largely separated the start of the drought (day 1) from the other time points (Fig. 1). CDF1 correlated positively with the monoterpenes α-pinene ($r = 0.42$, day effect in randomized block ANOVA, $P = 0.0069$) and β-ocimene ($r = 0.34$, $P = 0.0008$) and the sesquiterpene (*E,E*)-α-farnesene ($r = 0.34$, $P = 0.0021$), all of which had the lowest average emissions at the start of the drought. The highest negative correlations of CDF1 were with the benzenoid benzyl alcohol ($r = -0.55$, $P = 0.0049$) and the aliphatic compound 1,3-octadiene ($r = -0.35$, day effect in randomized block ANOVA, $P = 0.0287$). Emissions when plants were most drought stressed (day 12–13) differed from those during the middle of the drought (day 8–10) primarily in a direction correlated with emission of (*E*)-β-ocimene (Fig. 1).

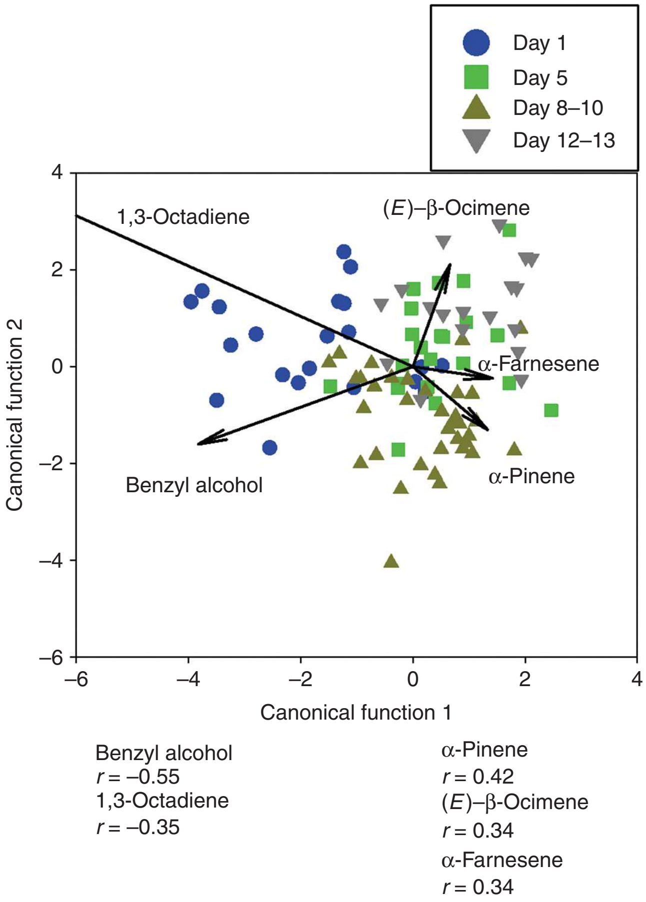

Fig. 1. Canonical discriminant analysis separating volatile emissions across the four time points in the drought. The progression of the drought from start (day 1) to late drought (days 12–13) is indicated by colour and symbol type. Compounds with the largest positive and negative correlations (r) with canonical discriminant function 1 (CFDF1) are labelled along the axes, with their loadings shown by arrows. CDF1 was highly correlated with group membership (canonical correlation $r = 0.70$, $P = 0.0037$), whereas CDF2 was not ($r = 0.67$, $P = 0.0823$).

The total amount of floral volatiles emitted generally increased during the artificial drought, with the mean amount during late drought (days 12–13), when soil moisture averaged 2.5 % vwc, significantly higher than that for day 1, when soil moisture averaged 16.6 % (Tukey comparison $P < 0.05$; Table 1). The proportion represented by α-pinene changed during the drought (day effect in randomized block ANOVA, $P = 0.0002$), first increasing and then declining again during late drought, with its peak after 8–10 d of drought (Tukey $P < 0.05$ for comparison of that time point with the start and end of the drought; Table 1) when soil vwc averaged 4 %. Of the six compounds that showed changes in emissions during the drought according to univariate randomized block ANOVA (Table 1), four had emissions detectably correlated with soil vwc [α-pinene, (*E,E*)-α-farnesene, benzyl alcohol and 1,3-octadiene]. Both α-pinene and (*E,E*)-α-farnesene showed highly increased variance in emissions as the soil dried out (Fig. 2), but that pattern was not seen for benzyl alcohol. The shape of the non-parametric regression (Fig. 2) indicated that emission of α-pinene first increased with moderate drought, peaked when the soil moisture was approx. 4 %, and then began to fall again. Emissions of (*E,E*)-α-farnesene stayed low through the first stages of the drought and then rose rapidly, with peak values seen under the driest conditions (Fig. 2). The shapes of the relationships are more difficult to characterize for benzyl alcohol and 1,3-octadiene because their overall emissions were low, each accounting for <1 % of the scent mixture (Supplementary Data Table S1).

Both photosynthetic rate (A) and stomatal conductance (g_s) showed curvilinear relationships with soil moisture (linear regression term, $P < 0.0001$ for both measures, quadratic regression term, $P = 0.0015$ and <0.0001, respectively). F_2 hybrids had higher rates of carbon assimilation and stomatal conductance, controlling for soil moisture ($P < 0.0001$ for both measures). With drier soil, both gas exchange measurements increased and then declined again (Fig. 3). For all plants combined, the optimal soil moisture for the photosynthetic rate was 18 % vwc, near the average moisture on day 1 of the drought (16.6 %), indicating that most plants were probably experiencing little drought stress at that point. Throughout most of the drought, the percentage emission of the dominant scent compound α-pinene increased steadily and only declined again on day 13 (Table 1). At that point, soil moisture content had fallen to an average of 2.5 %, a value at which stomatal conductance was very low and the photosynthetic rate averaged only 33% as high as on day 1 (Fig. 3), indicating

Table 1. *Changes in mean values for floral volatiles over the course of the drought*

Compound	Start: day 1	Early: day 5	Mid: days 8–10	Late: days 12–13
% α-Pinene	32.8^{a}	47.5^{ab}	55.3^{b}	35.4^{a}
α-Pinene (ng h^{-1})	17.5^{a}	$45.9^{a,b}$	44.3^{b}	38.5^{b}
(*E*)-β-Ocimene (ng h^{-1})	4.2^{a}	6.9^{a}	5.8^{a}	20.9^{b}
β-Pinene (ng h^{-1})	0^{a}	1.4^{a}	0^{a}	0.5^{a}
(*E,E*)-α-Farnesene (ng h^{-1})	0.8^{a}	3.6^{ab}	2.8^{ab}	8.6^{b}
1,3-Octadiene (ng h^{-1})	0.07^{a}	0^{ab}	0^{b}	0.02^{ab}
Benzyl alcohol (ng h^{-1})	1.2^{a}	0.3^{b}	0.5^{b}	0.3^{b}
Total (ng h^{-1})	54.5^{a}	85.1^{ab}	77.1^{ab}	117.0^{b}

Values are reported for absolute emissions of the six volatiles that changed detectably over the drought based on an overall $P < 0.05$ in a randomized block ANOVA, the total emissions and the percentage comprised by α-pinene. Absolute emissions of individual volatiles were log-transformed prior to analysis.

Values with different superscripts differ significantly ($P < 0.05$) based on Tukey post-hoc comparisons. Total sample size = 97.

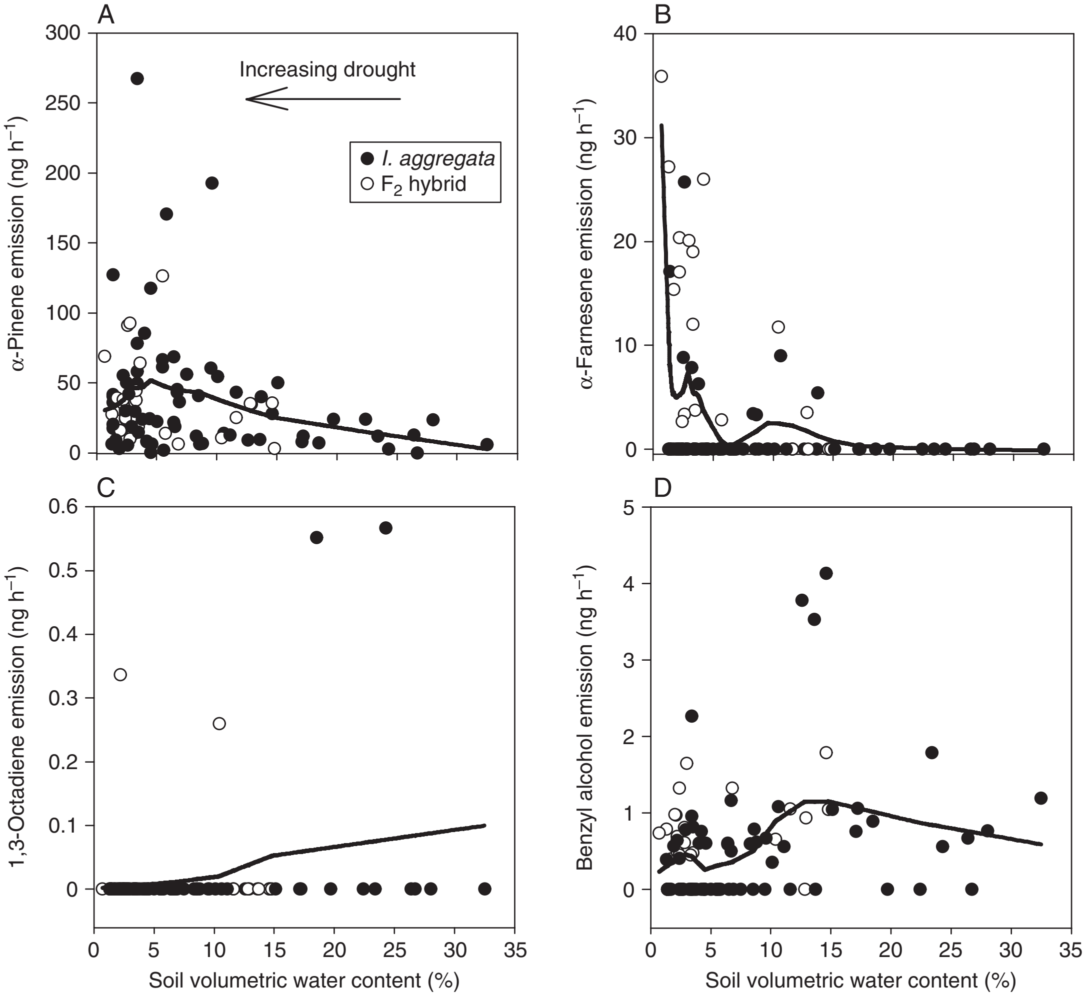

FIG. 2. Volatile emission rates per flower as a function of soil moisture. Lines were fit using a non-parametric regression smoothing method that minimizes the AICC criterion.

high plant stress. Following rewetting of plants in 2015, mean photosynthetic rate rebounded to 81 % of its value on day 1 that year, indicating that plants were able to recover from the level of drought stress applied. We detected no interactions between day and photosynthetic rate on total emissions (P = 0.28), or individually for any of the four compounds that changed with soil moisture (all $P > 0.40$ after false discovery rate correction for four volatiles), nor any main effects of photosynthetic rate in standard ANCOVAs (all $P > 0.15$). Total volatile emission increased with stomatal conductance (standard ANCOVA, $P = 0.0214$). That effect was due to the positive relationship evident at the start on day 1 before plants were drought stressed (Supplementary Data Fig. S1). None of the individual compounds showed detectable responses to stomatal conductance (all $P > 0.25$ for interactions between day and conductance and all $P > 0.10$ for main effect after removal of the interaction). As total emissions increased steadily during the drought, they did not show the overall pattern of an increase followed by a decrease seen for gas exchange levels. Thus, floral volatile emissions did not track closely the time course of changes in carbon assimilation or stomatal conductance.

DISCUSSION

Changes in volatiles with soil moisture

An experimentally imposed reduction in soil moisture caused a shift in composition of floral volatiles, from more of the aliphatic compound 1,3-octadiene and the benzenoid benzyl alcohol to higher representation of the monoterpenes α-pinene and (*E*)-β-ocimene and the sesquiterpene (*E,E*)-α-farnesene. The responses occurred across a range of average experimental soil moistures (2.5–16.6 %) similar to ranges recorded from nearby natural populations of these *Ipomopsis* species (Campbell and Wu, 2013; Waser and Price, 2016). Along with Burkle and Runyon (2016), our findings for *Ipomopsis* add volatiles to the set of floral traits observed to show phenotypic plasticity to drought. Prolonged drought can reduce nectar production (Waser and Price, 2016) and the length of the corolla tube in *I. aggregata* (Campbell and Wendlandt, 2013), and similar responses of nectar and flower size are well known from natural populations of a range of plant species (Carroll *et al.*, 2001; Caruso, 2006; Gallagher and Campbell, 2017). As for these other floral traits, the changes in volatiles

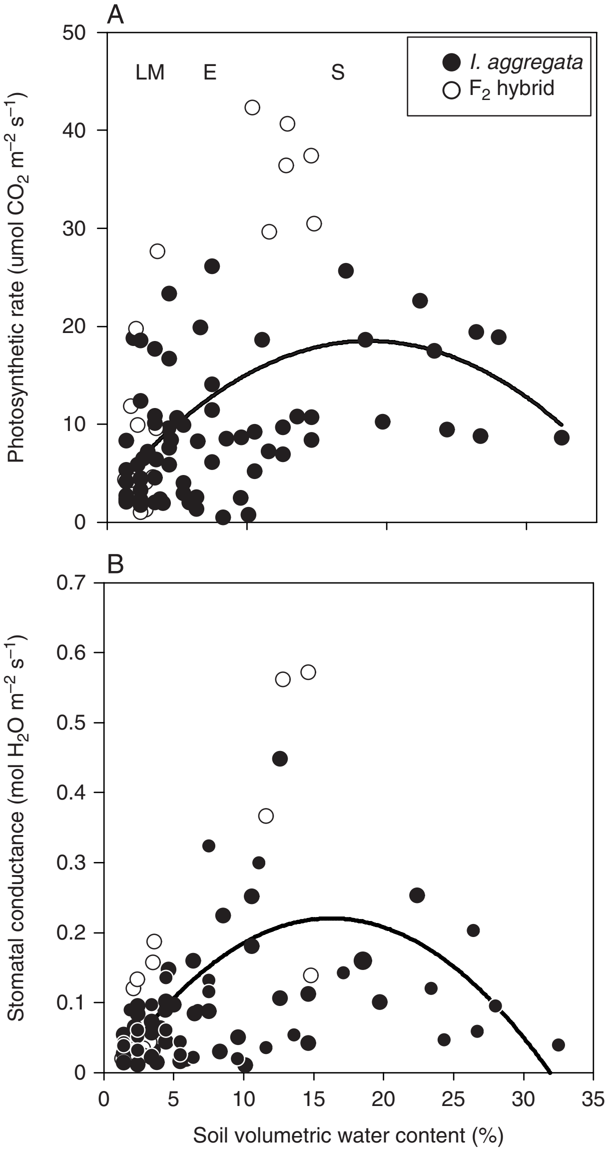

FIG. 3. Photosynthetic rate and stomatal conductance as a function of soil moisture. Lines depict best-fitting quadratic relationships. Top: letters S, E, M and L indicate the mean water content at the start (day 1), early (day 5), mid (days 8–10) and late (days 12–13) time periods, respectively, during the drought.

have the potential to alter the behaviour of pollinators or seed predators.

Our study furthermore demonstrates that some floral volatile emissions change in a non-linear fashion with soil moisture, such that responses to extreme drought may not be predictable from responses to milder drought. For example, emissions of α-pinene, the major constituent of floral scent in *Ipomopsis*, decreased again as soil moisture dropped from 4 % to 2.5 % vwc, when plants were strongly drought stressed, with stomatal conductance well below half of its maximum. It represented the highest proportion of the overall scent mixture during mid-drought (days 8–10). In contrast, emissions of (*E,E*)-α-farnesene showed an accelerating increase as the drought intensified. Flowers from two of the four previously studied species also showed a trend towards increased α-pinene emission with one imposed level of drought (although statistical testing was not reported for individual compounds due to small sample sizes), but not consistently so for *Heterotheca villosa* or *Phacelia hastata* (Burkle and Runyon, 2016; Glenny *et al.*, 2018). Such species-specific responses to a single drought level might be explained by non-linear responses to soil moisture, as it is possible that soil moisture experienced by *H. villosa* put it on the part of the curve where emissions were declining again. For three out of four of these other plant species studied, benzyl alcohol emissions appeared either constant or declined with drought, just as in *I. aggregata*. Most other compounds that responded in *I. aggregata* were not emitted by flowers of these other species, although (*Z*)-3-hexen-1-ol showed either an increase or no change with drought (Burkle and Runyon, 2016), in contrast to the decrease seen for (*E*)-3-hexen-1-ol in *I. aggregata*. The compounds that showed increased emissions with drought in *Ipomopsis*, monoterpenes and a sesquiterpene, are synthesized through biosynthetic pathways different (methylerythritol and mevalonic acid pathways, respectively) from those of benzenoids and green leaf volatiles (Dudareva *et al.*, 2013). It would be valuable to know more generally if floral VOCs produced through different pathways respond differentially to drought. Interpretation of responses would also be aided by knowledge of the tissues that emit the floral volatiles. These *Ipomopsis* plants have trichomes on all green tissues including floral calyces, which might emit some of the floral compounds, as glandular trichomes can be common sites of synthesis, storage and secretion of terpenes, as well as other compounds (Schilmiller *et al.*, 2008; Gonzales-Vigil *et al.*, 2012). While it is conceivable that nectar, rather than petals or sepals, emits some of the volatiles in *Ipomopsis*, it is unlikely that changes in nectar production could be rapid enough to explain the observed changes over 13 d in volatile emissions. Only one of four studies (Campbell and Halama, 1993; Burkle and Irwin, 2009; Campbell and Wendlandt, 2013; Waser and Price, 2016) detected effects of manipulating water availability on nectar production in *I. aggregata*, and that study began the water manipulations prior to production of the first flowers in the season (Waser and Price, 2016).

Plants of *I. aggregata* and hybrids with its closest congener *I. tenuituba* showed similar responses of volatiles to drought, increasing the generality of the results. However, the F_2 hybrids we tested differed in scent composition. Previous studies of hybrids between these species relied upon natural hybrids of unknown genetic background, as have most studies of floral volatiles in hybrids (but see studies of *Petunia* by Klahre *et al.*, 2011 and others). The natural hybrids sampled *in situ* emitted less α-pinene and β-myrcene than did *I. aggregata* (Bischoff *et al.*, 2014), which was not the case for the F_2 hybrids studied here. Instead they emitted more cadinene, (*E,E*)-α-farnesene, linalool and α-ylangene than did *I. aggregata*. Interpreting these differences is difficult, as the hybrids in both studies experienced vegetative growth under conditions different from the *I. aggregata* plants. Further studies of the genetics of these volatile emissions will be necessary to elucidate changes in emissions in hybrids. Although there was high overlap in VOCs detected here and in the earlier study of *Ipomopsis* (Bischoff *et al.*, 2014), and both studies found α-pinene to be the dominant volatile, some compounds detected in the earlier study were not detected in this one, and three new compounds were detected (Supplementary Data Table S1). Two of these three compounds averaged higher emissions from the F_2 hybrids, which were not previously studied. Differences between studies could also be due to the use of potted vs. *in situ* plants, potentially associated with differences in soil or endophytic microbes, as bacteria on plant tissues can alter volatile emissions (Junker and Tholl, 2013). Alternatively, they may result from the use of Carbotrap as well as Tenax TA® as trapping compounds (Bischoff *et al.*, 2014). For this study, we sampled volatile emissions only during the day. Emission of a hawkmoth attractant, indole, that influences reproductive isolation is ramped up during the hours after dusk (Bischoff *et al*., 2014, 2015), so it would be interesting to extend this work to consider how drought influences night-time emissions.

During the experimentally imposed drought, carbon assimilation and stomatal conductance peaked near a soil moisture of 18 % vwc and then declined thereafter. Thus plants were not in drought stress on the first day that water was withheld but probably were at all other sampling points. That relationship to soil moisture was similar to results obtained for *I. aggregata* in a previous dry-down study, in which carbon assimilation peaked at 17 % vwc (Campbell *et al.*, 2010). The levels of gas exchange were not closely coupled to emissions of any particular floral volatile. Also total emissions were positively correlated with stomatal conductance only on day 1 when plants were not yet drought stressed. Those results contrast with leaf emissions of some monoterpenes, which can be coupled with gas exchange (Nogues *et al.*, 2015), but particularly so under very severe drought conditions (Eller *et al.*, 2016), in some cases due to reduced *de novo* biosynthesis when carbon assimilation is sufficiently inhibited (Niinemets, 2010). For leaves, water-soluble compounds can also show strong sensitivity to stomatal closure due to changes in gas phase diffusion (Niinemets *et al.*, 2004).

Implications for spatial, seasonal and long-term climatic changes

The changes with soil water mean that signatures of floral volatiles are likely to vary across habitats and across time. Thus variation in soil water is one aspect of the environment that could be responsible for some of the large variation in floral volatiles often reported within or across populations of a given plant species (Delle-Vedove *et al.*, 2017). *Ipomopsis aggregata* subsp. *aggregata* in particular is widespread throughout the mountains of the western USA (Grant and Wilken, 1986). Habitats include openings in coniferous forest and sagebrush-dominated meadows, as well as sub-alpine meadows, with an elevational range from 600 to 3400 m a.s.l. (Grant and Wilken, 1986). Soil moisture is likely to vary greatly across such a range, as described by Campbell and Wu (2013), suggesting that floral scent blends in *Ipomopsis* could change from population to population simply for environmental reasons. At this point, we do not know how the extent of plasticity to such environmental conditions compares with the level of genetic variation in scent emissions across populations or heritability within populations, about which information remains scarce (Zu *et al.*, 2016).

In sub-alpine meadows in Colorado, where we have studied these plants most intensely, soil moisture also changes seasonally, with plants experiencing an early summer drought between snowmelt (typically during May) and arrival of the summer monsoon rain in early July (gothicwx.org). During late June, soil moisture usually falls below 10 % vwc (Campbell and Wu, 2013; Waser and Price, 2016). Soil moisture can drop as low as 2 % (Waser and Price, 2016) or even lower, as seen in 2018 (D. Campbell, pers. obs.), but outside of those extreme drought situations, in the early summer plants probably often experience conditions when emissions of α-pinene should be relatively high on average but also highly variable. Total scent emitted is also expected to be high during June for plants growing in these relatively dry soils. Once the summer monsoons start, plants are likely to decline in overall scent and particularly in emission of α-pinene if rains are sufficient to increase soil moisture well above 4 % vwc. This seasonal shift means that olfactory signals presented to potential pollinators and herbivores are likely to differ over the season, as seen for several Mediterranean plant species (Farré-Armengol *et al.*, 2014). It would be valuable to study how those shifts alter both pollination by hawkmoths in *Ipomopsis* (Campbell *et al.*, 1997) and seed predation by the anthomyiid fly *Delia* sp., for which a single larva consumes all of the seeds in a fruit (Brody, 1992). Hawkmoth pollinators typically arrive late in the summer at our sites, if at all, whereas hummingbird pollinators and the fly seed predators span the majority of the *Ipomopsis* flowering season (Campbell *et al.*, 1997). The compound α-pinene can be learned by noctuid moths in association with nectar (Cunningham *et al.*, 2004). Its presence also appears to decrease fitness in a moth-pollinated orchid, possibly by interacting with attractiveness of other compounds in the scent blend (Schiestl *et al.*, 2011). That compound also stimulates oviposition and increases longevity in a tephritid fly that feeds on olive (Gerofotis *et al.*, 2016). The effect of augmenting α-pinene on flower bud oviposition by the fly *Delia* sp. is currently under investigation in *I. aggregata*.

The observed changes in floral volatiles with soil moisture also suggest that global climate change will alter the scent bouquets. Projections are for increased temperatures, earlier snowmelt (Wadgymar *et al.*, 2018) and reduced soil moisture in parts of the Colorado Rockies where these plants are common (Seager *et al.*, 2012; IPCC, 2014). Although we do not yet know the responses of specific floral volatiles to temperature in these species, investigations of other species have often found increases in monoterpene or total terpene emissions with rising temperature (Hansted *et al.*, 1994; Farré-Armengol *et al.*, 2014), broadly consistent with general models for terpenoid emission in plants (Niinemets *et al.*, 2004). If similar responses are seen in *Ipomopsis*, we could expect those temperature responses to reinforce the already higher levels of monoterpenes expected under moderately drier conditions. Our demonstration of floral volatile responses to soil moisture reinforces the suggestion that climate change may alter both the total amount and composition of floral scent blends.

Conclusion

Here we measured for the first time the quantitative response of floral volatile composition to soil moisture. Our results indicate that variation in water availability is one source of environmental variation in these traits that often influence interactions with pollinators and herbivores. Moreover, the non-linear responses of emissions to volumetric water content suggest that volatile responses to severe droughts may not be predictable from responses to milder droughts.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: relative amounts of daytime floral emissions (mean percentage of total scent blend averaged over all floral samples). Table S2: comparisons of daytime volatile emissions by *Ipomopsis aggregata* and the F_2 hybrids. Figure S1: total VOC emission as a function of stomatal conductance during the four time periods of the drought.

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